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**{Exhibit 36}**

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## A RAPID AND SENSITIVE IMMUNOLOGICAL METHOD FOR IN SITU GENE MAPPING<sup>1</sup>

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**ABSTRACT** A method for in situ localization of specific DNA sequences has been developed which exploits the interaction between modified nucleotides and antibodies directed against the modification. Cloned sequences of *Drosophila* melanogaster DNA are nick translated in vitro in the presence of *E. coli* DNA polymerase I and an analogue of dUTP which contains a biotin molecule covalently linked to the C-5 position of the pyrimidine ring. The nick translated probe, with approximately 1-5% of its nucleotides containing biotin, is hybridized in situ, according to standard protocols, to *Drosophila* salivary gland chromosomes. After hybridization, the slides are incubated with monospecific rabbit anti-biotin immunoglobulins followed by FITC-goat anti-rabbit IgG. After counterstaining with Evans Blue, fluorescent yellow-green bands, corresponding to the map location of the cloned DNA are seen against a red fluorescent background of the salivary gland chromosomes. Methods for refining the system for use in localization of unique sequences on mammalian metaphase chromosomes are also discussed.

## INTRODUCTION

The mapping of genes or their transcripts to specific loci on chromosomes has proven to be a tedious and time consuming occupation, mainly involving techniques of cell-fusion and somatic cell genetics. Although in situ hybridization has been employed successfully for mapping single-copy gene

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sequences in species that undergo chromosome polyploidization, such as *Drosophila*, detection of unique sequence genes in most higher eukaryotic chromosomes has been extremely difficult (1). The necessity for polynucleotide probes of very high specific radioactivity to facilitate autoradiographic localization of the hybridization site results in rapid radiodecomposition of the probe and a concomitant increase in the background noise of silver grain deposition. The use of hybridization probes with low to moderate specific radioactivities requires exposure times which are often impractical. The objective of this study was to test the feasibility of a novel approach to *in situ* hybridization which circumvents the radiochemical and temporal limitations of the normal hybridization methods, and which offers the potential of rapid and specific analysis of single-copy sequences. This approach exploits the interaction between a modified nucleotide and antibodies directed against this modification.

Biotin has many features which make it an ideal modification probe (2). Davidson and his colleagues (3-5) took advantage of the biotin-avidin interaction by chemically crosslinking biotin to RNA via cytochrome C or polyamine bridges. Sites of hybridization were then detected in the electron microscope through the binding of avidin-ferritin or avidin-methacrylate spheres. Although successful in the specialized cases examined, this method has not proven to be of general utility. It is likely that this is due to the nonspecific interaction of avidin with DNA or chromatin which we and others (2,6,7) have observed. To circumvent this limitation we have developed immunological reagents for biotin detection which do not exhibit nonspecific binding to chromosomes and have used these in conjunction with biotin labeled nucleotides which can be enzymatically incorporated into a hybridization probe.

#### METHODS

**Synthesis of Biotinyl-Deoxyuridine 5'-Triphosphate.** An analogue of dUTP that contains a biotin molecule covalently bound to the C-5 position of the pyrimidine ring through an allylamine linker arm was synthesized according to the scheme illustrated in Figure 1. Allylamine-dUTP was prepared from 5-mercuri-dUTP (8) by reaction with a 10-fold molar excess of allylamine and one nucleotide equivalent of potassium tetrachloropalladium ( $K_2PdCl_4$ ) in 0.25 M sodium acetate buffer, pH 5.0 for 18-24 hours. Similar palladium-catalyzed alkylation reactions have been used previously for the synthesis of a variety of C-5 substituted pyrimidine nucleoside compounds (9,10). The allylamine-dUTP product was purified by ion

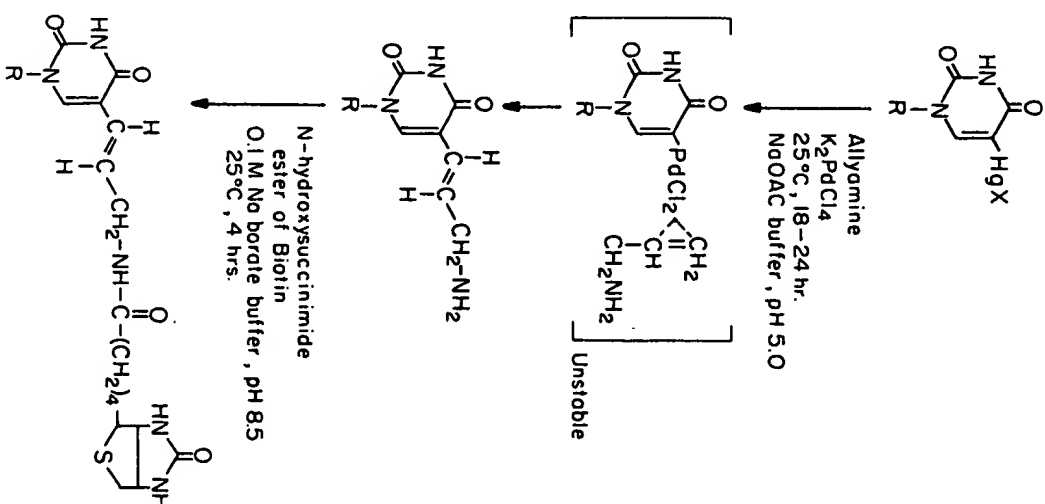


FIGURE 1. Reaction scheme used for synthesis of the biotinylallylamine-derivative of dUTP (Bio-dUTP).

exchange chromatography on DEAE-cellulose and by HPLC reverse phase chromatography on a Partisil ODS-2 support. The biotinylallylamine-derivative of dUTP (Bio-dUTP) was prepared by reaction with two equivalents of biotinyl-N-hydroxysuccinimide ester in 0.1 M sodium borate buffer, pH 8.5, for 4 hours at room temperature and purified by DEAE-cellulose chromatography. Details on the synthesis, purification and characterization of Bio-dUTP will be presented elsewhere (11).

**Preparation of Rabbit Anti-biotin Antibodies.** Biotin-BSA, prepared as described (12), was used to raise antibody in rabbits. The immunization schedule was modified from that used by Berger (13) and is detailed elsewhere (manuscript in preparation). The animals were immunized initially with 1.2 ml of antigen (2 mg/ml biotin-BSA in 0.9% NaCl diluted with an equal volume of complete Freund's adjuvant) and boosted every 14 days with 1 ml of 0.9% NaCl containing 2 mg biotin-BSA. Within 6 weeks after immunization, the animals were producing anti-biotin antibodies as determined by ouchterlony immunodiffusion. Beginning at this time, 30-40 cc of blood was collected 10-11 days after each boost. The blood was incubated at 37°C for 1-2 hours, and then refrigerated at 4°C for a minimum of 4 hours. Serum was separated from clotted material by centrifugation and stored at -20°C.

**In Situ Hybridization.** Cloned *Drosophila* DNA, nick translated in the presence of  $^3\text{H}$  dATP and either TTP or Bio-dUTP, was hybridized to *Drosophila* polytene chromosomes as described (14). The acetylation step described by Hayashi et al. (15) was included before denaturation of the chromosomes (personal communication, M. Pardee). After hybridization, non-specifically bound material was removed by washing in 2X SSC at 60°C and then at room temperature. The slides were then rinsed in PBS and incubated with rabbit anti-biotin (2.5 µg/ml in PBS + 10 mg/ml BSA) at 37°C. The slides were again rinsed in PBS and the chromosome spreads were incubated with FITC-goat anti-rabbit IgG (Miles, diluted 1:100 in PBS + 10 mg/ml BSA). After rinsing in PBS, the chromosomes were counterstained for 2 minutes with Evans Blue (0.5% w/v in PBS + 1% fetal calf serum), rinsed in PBS, mounted in 0.1 M Tris pH 8.0, 90% glycerol, and viewed with an epi-illuminated phase-fluorescent microscope.

## RESULTS

### Preparation of Biotin-substituted Hybridization Probes.

Bio-dUTP can replace TTP as a substrate for a variety of DNA polymerases of both prokaryotic and eukaryotic origin

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(11). Similarly, the ribonucleotide analogue, Bio-UTP, can substitute for UTP in reactions catalyzed by the RNA polymerases of *E. coli* and bacteriophage T7 (11). Thus, biotinyl-DNA and biotinyl-cRNA hybridization probes can be synthesized conveniently and rapidly by straightforward enzymatic reactions. The substrate properties of Bio-dUTP with *E. coli* DNA polymerase I, using either the nick-translation protocol of Rigby et al. (16) or the gap-filling reaction of Bourguignon et al. (17), are illustrated in Figure 2. Although the

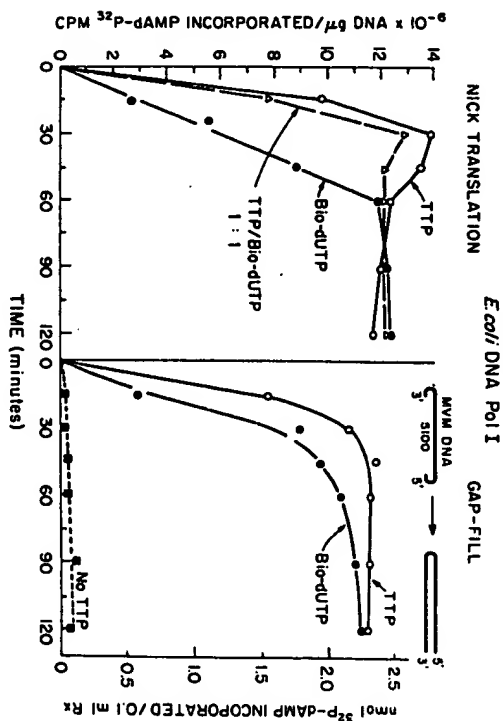


FIGURE 2. Bio-dUTP is a substrate for *E. coli* DNA polymerase I. (A)  $\lambda$  phage DNA was nick translated in vitro with DNA polymerase I holoenzyme as described by Rigby et al. (16). Reactions were done in the presence of  $^{32}\text{P}$ -dATP (1.35 µM, 400 Ci/mmol) and either TTP (20 µM), Bio-dUTP (20 µM), or an equal concentration of TTP and Bio-dUTP (10 µM each). (B) Minute Virus of Mice (MVM) DNA, a 5 Kb single-stranded DNA molecule with terminal hairpin duplexes (20), was converted to double-stranded (RF) DNA by reaction with DNA polymerase I (Klenow fragment) as described by Bourguignon et al. (17). The three nucleotide reaction contained only 0.1 mM of dCTP, dGTP and  $^{32}\text{P}$ -dATP (50 µCi/µmole). TTP or Bio-dUTP reactions were supplemented with the appropriate triphosphate at a final concentration of 0.1 mM.

analogue is incorporated at initial rates which are only 30-40% that of the control (TTP-containing) reactions, the final specific activities (and the extent of polymerization) that can be achieved are essentially the same.

The  $T_m$  of biotinated polynucleotide duplexes decreases as the biotin-content of the polymer increases (11). However, DNA in which every TMP residue in one strand is replaced by a Bio-dUMP residue (e.g., Bio-MWM RF DNA in Figure 2), has a  $T_m$  value that is only 5°C less than that of unsubstituted control DNA. Furthermore, nick translated DNA probes that have between 0.2 and 2.0% of their total nucleotides biotinized exhibit reassociation rates that are essentially the same as those observed with biotin-free DNA (11). Since a substantial number of biotin molecules can be introduced into a polynucleotide without significantly altering its hybridization characteristics, the same *in situ* hybridization conditions have been used for both the control and biotin-substituted DNA probes employed in the experiments described below.

Purification of Anti-biotin Antibodies by Affinity Chromatography. Biotin specific antibodies from immune serum (see Methods) were purified by affinity chromatography on ovalbumin sepharose and biotin-ovalbumin sepharose. The resins were prepared by coupling ovalbumin or biotin-ovalbumin (12) to cyanogen bromide activated sepharose 4B (18). Serum was loaded onto ovalbumin sepharose to remove any ovalbumin binding component. The flow through from this column was loaded directly onto biotin-ovalbumin sepharose. This resin was washed extensively with phosphate-buffered saline (PBS) until the flow through contained no detectable protein. Protein specifically bound to the resin was then eluted with 3 M KSCN in PBS, concentrated in an amicon filter unit, and desalted on a Sephadex G-25 column equilibrated in PBS. The antibody recovered in the void volume was adjusted to a concentration of  $\approx 0.5$  mg/ml in PBS with 5-10 mg/ml BSA, and stored at -20°C.

This antibody was assayed for specificity against biotin-labeled DNA by immunoprecipitation with Staph aureus (19). DNA, nick translated with  $^{32}\text{P}$ -dATP and either TTP or Bio-dUMP, was used in these assays. When unmodified DNA was incubated in the absence of serum, with nonimmune serum, or with rabbit anti-biotin the  $^{32}\text{P}$  counts remained in the supernatant. Bio-DNA, either in the absence of serum or in the presence of nonimmune rabbit serum also remained in the supernatant. However, Bio-DNA was immunoprecipitated with affinity purified rabbit anti-biotin in the presence of Staph A protein. The percentage of the total Bio-DNA radioactivity found in the immune precipitate was dependent upon the

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antibody concentration and the time of incubation with the antigen. Under optimum conditions, greater than 90% of the Bio-DNA is immunoprecipitable.

In Situ Hybridization. A variety of cloned Drosophila sequences were used to test the specificity and sensitivity of the immunological method of hybrid detection. Results obtained with two specific clones, designated pW 539 and pAC 104, will serve to illustrate the potential advantages of this procedure over conventional autoradiographic detection. Clone pW 539, obtained from Otto Schmidt (Yale University), contains a 22 kb fragment encoding a methionine tRNA gene inserted into a pMB9 plasmid (S. Sharp et al., manuscript in preparation). This sequence is known to map to band 61D on chromosome 3L. Clone pAC 104, obtained from V. Pirrotta (EMBO), contains 6 kb of a transposition element inserted into the plasmid pACYC 184 (V. Pirrotta, personal communication). This cloned fragment maps to numerous loci throughout the Drosophila genome. The probes were nick translated *in vitro* with  $^3\text{H}$  dATP and either TTP or Bio-dUMP. Kinetics of Bio-dUMP and TMP incorporation were similar in both cases to those illustrated in Figure 2. These probes were then hybridized to chromosomes *in situ* for 10-12 hours at 65°C (14,15). After removal of nonspecifically bound material, the slides were washed and incubated with antibodies as described in the methods section. A reasonable fluorescent signal was obtained with 1 hour incubations of both antibodies, but the optimum time for a strong signal was determined to be 4 hours for the first antibody and 2 hours for the FITC-goat anti-rabbit IgG. Figures 3 and 4 are sets of phase, immunofluorescent, and autoradiographic exposures of the same chromosome spreads.

Figure 3, showing hybridization of a unique Drosophila sequence (clone pW 539) establishes the clarity and specificity of the signal obtained by immunofluorescence. Hybridizations using  $^3\text{H}$  labeled -- but Biotin-free -- DNA followed by antibody staining, showed no detectable fluorescent signal even though autoradiographic exposures indicated that accurate and efficient hybridization had occurred.

Figure 4, illustrating hybridization of the clone pAC 104, shows the tremendous increase in resolving power offered by this technique. This is easily seen by a comparison of regions indicated by the arrows in the fluorescent and autoradiographic exposures. The number of bands at these sites are difficult to determine from the autoradiogram, however, they are easily counted in the immunofluorescent picture.

Overall, the immunological method of hybrid localization offers four immediate advantages over autoradiography. These include a decrease in the time required for localization,

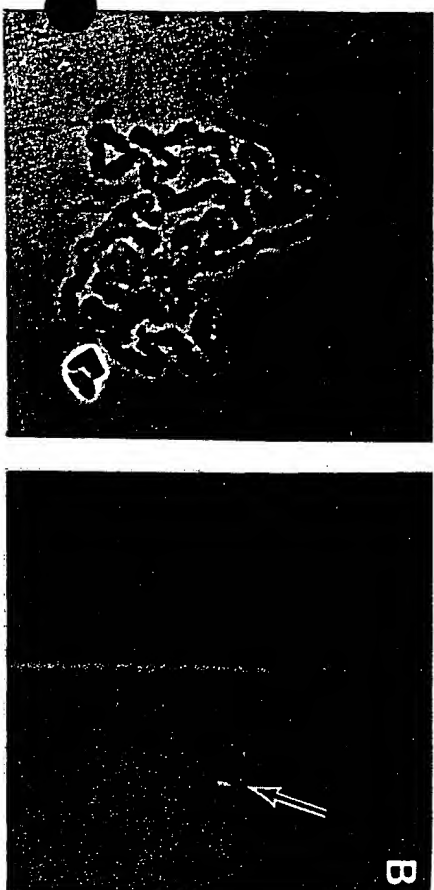


FIGURE 3. Results of in situ hybridization using clone PW 539 DNA that contained both  $^3\text{H}$ -dAMP (8.8 x  $10^5$  cpm/ $\mu\text{g}$ ) and Bio-dUMP (7% of TMP residues substituted). (A) phase picture, (B) immunofluorescence picture, (C) autoradiographic exposure (9 days).

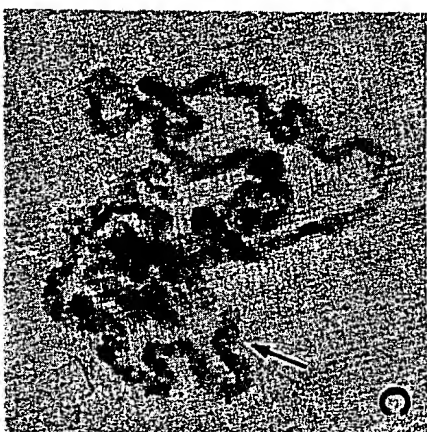
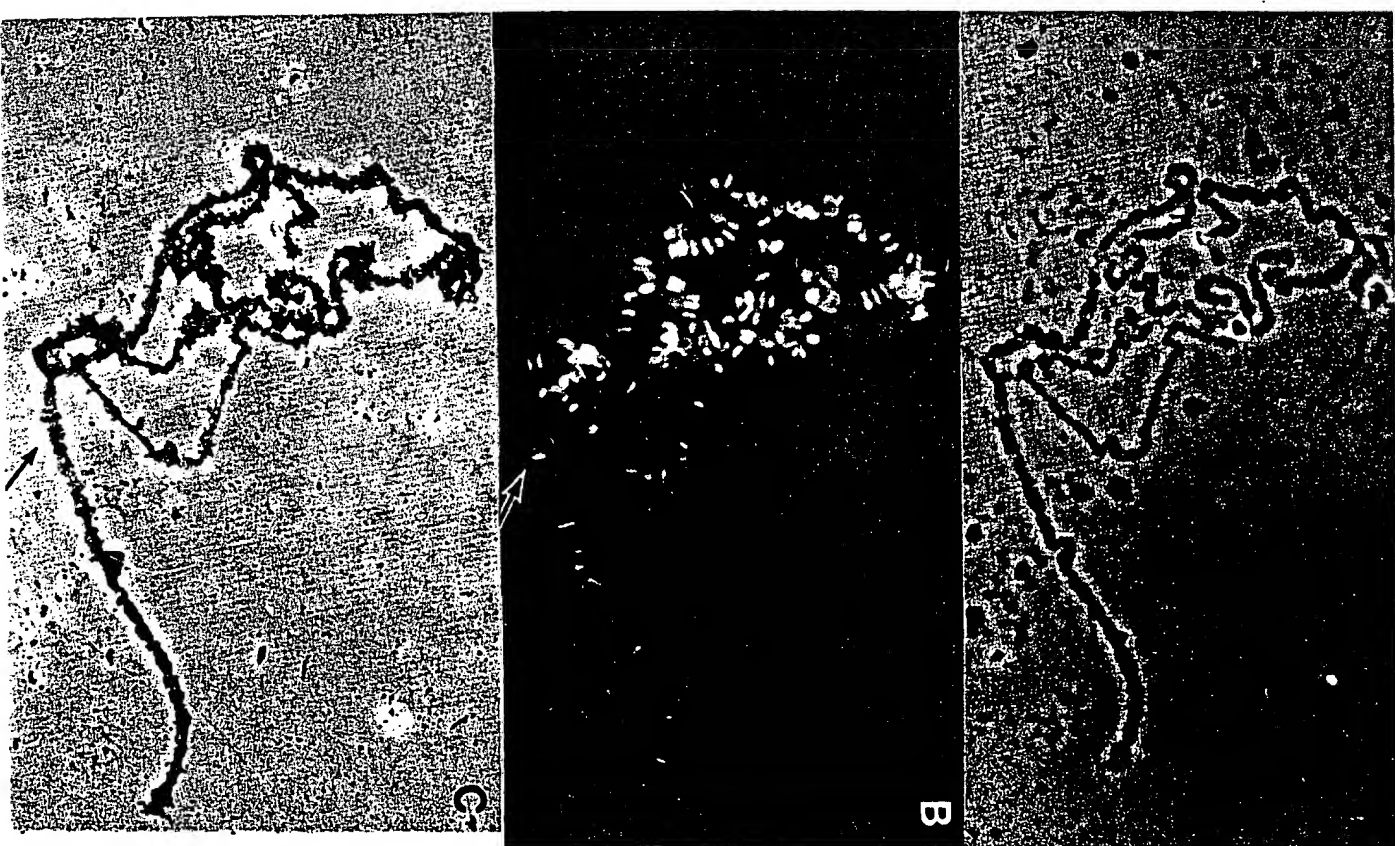


FIGURE 4. Results of in situ hybridization using clone PAC 104 DNA that contained both  $^3\text{H}$ -dAMP (5 x  $10^6$  cpm/ $\mu\text{g}$ ) and Bio-dUMP (17% of TMP residues substituted). (A) phase picture, (B) immunofluorescence picture, (C) autoradiographic exposure (11 days).

improved resolution, a lower background noise and the ability to prepare stable hybridization probes which are not subject to the decomposition problems found with radiolabeled probes.





## DISCUSSION

The results outlined above demonstrate that the interaction between biotinyl-nucleotides and anti-biotin antibodies can be used effectively for the *in situ* localization of DNA sequences within the *Drosophila* polytene chromosome. The fluorescent bands observed in Figures 3 and 4 represent the signal produced by about 100-200 copies of biotinized DNA probes that can hybridize to 6-22 Kb of *Drosophila* DNA. This estimate comes from the polytenization number of 500 and a hybridization efficiency to alkali-denatured chromosomes of 20-40% (21). Additional studies (Langer and Ward, manuscript in preparation) have shown that bands with fluorescent signals only 5-10% as strong as those illustrated here can be visualized and photographically recorded. The latter observation suggests that the simple "antibody-sandwich" method described here is sensitive enough to detect a chromosomal locus which hybridizes 10-20 copies of a sequence a few kilobases in size or 100-200 copies of a sequence a few hundred nucleotides long.

This immunological method of hybrid detection is not restricted to *Drosophila* polytene chromosomes since we have used it successfully to determine the chromosomal loci of reiterated sequences, e.g., satellite DNA's, on mammalian metaphase chromosomes (Langer and Ward, manuscript in preparation). In addition, Bio-CRNA probes have been used in conjunction with anti-biotin antibodies to localize the histone gene clusters on the lampbrush chromosome of the Newt, *Notophthalmus* (J. Gall, personal communication). Nevertheless, further refinements of the basic method will be required before single-copy sequences can be visualized on mammalian metaphase chromosomes. Fortunately, there are a variety of approaches that can be used to either increase the fluorescent signal generated by the probe or to enhance the sensitivity of signal detection. For example, by using a "hapten-antibody sandwich" technique (22) the fluorescent signal can be enhanced by up to 50-fold over that achieved by the standard double-antibody method (23-24). Another approach to signal amplification is to treat the chromosomes alternatively with streptavidin and a biotinylated, FITC-labeled "carrier" protein. Streptavidin is a tetrameric protein produced by *Streptomyces avidinii* that binds four biotin molecules, each with a  $K_{dis}$  of  $10^{-15}$  (25). Since this protein, unlike avidin, does not exhibit non-specific binding to chromosomes (A. Chl, P. Langer and D. Ward, unpublished), one can hopefully exploit the high affinity of the streptavidin-biotin interaction to deliver many layers of the fluorescent "carrier" protein rapidly and specifically.

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One of the practical problems with weak fluorescent signals is that they often bleach rapidly, thus making it difficult to record the signal photographically. This limitation can be circumvented, however, by using low-light intensity illumination in conjunction with electronic image intensification. Alternatively, one can employ histochemical detection by coupling the primary or secondary antibody to enzymes, such as peroxidase, alkaline phosphatase, and  $\beta$ -galactosidase (26). The enzymatic conversion of soluble substrates to insoluble colored precipitates at the site of hybridization would also permit visualization by standard light microscopy. We are currently exploring a variety of such procedures to increase the sensitivity of probe detection with the objective of developing a protocol whereby single-copy sequences in mammalian chromosomes can be mapped with speed and precision.

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# A STRATEGY FOR HIGH-SPEED DNA SEQUENCING

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## I. HIGH-SPEED DNA SEQUENCING

Two DNA sequencing methods have been developed recently, one which involves chemical modification and cleavage of DNA (Maxam & Gilbert, 1977), and another which involves *in vitro* DNA synthesis using chain terminators (Sanger et al., 1977). Although 400 nucleotides can be read off a sequencing gel from a single reaction (Sanger & Coulson, 1978), these methods alone do not allow the rapid sequencing of long sequences. The time consuming steps in DNA sequencing are not the sequencing reactions but the prior steps: the isolation of single DNA fragments in small 400 nucleotide long pieces.

To obtain these fragments for the chemical method (Maxam & Gilbert, 1977) the DNA is cut by restriction endonucleases into smaller fragments, labeled at their ends with radioactive <sup>32</sup>P, cut with a second restriction endonuclease and the differently sized fragments purified by gel electrophoresis. Two problems arise. The number of DNA fragments generated by restriction endonucleases increases with the length of the DNA. Consequently the purification of DNA fragments by gel electrophoresis becomes impossible. In addition, for proof-reading both strands have to be sequenced. If the two strands